HERBICIDE- BINDING ACTIVITY OF THE PLASMA MEMBRANE FROM GRASS COLEOPTILES

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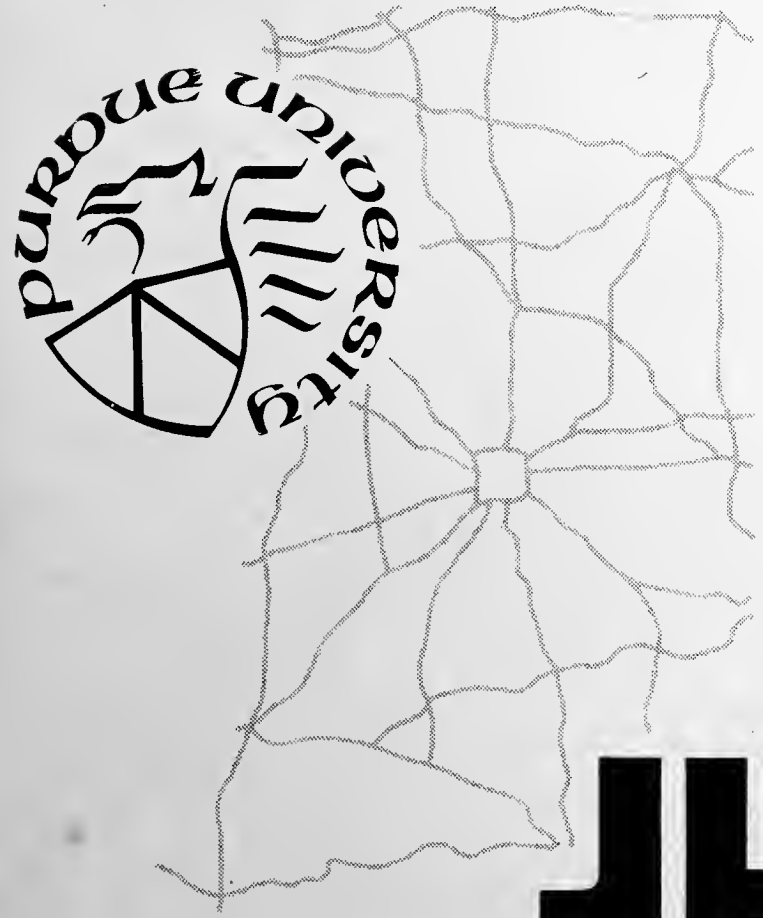

BY

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JOINT HIGHWAY RESEARCH PROJECT

PURDUE UNIVERSITY AND

INDIANA STATE HIGHWAY COMMISSION

Progress Report

HERBICIDE-BINDING ACTIVITY OF THE PLASMA MEMBRANE FROM GRASS COLEOPTILES

TO: J. F. McLaughlin, Director
Joint Highway Research Project

March 24, 1971

Project: C-36-48C

FROM: H. L. Michael, Associate Director
Joint Highway Research Project

File: 9-5-3

The attached Progress Report titled "Herbicide-Binding Activity of the Plasma Membrane from Grass Coleoptiles" is presented as partial fulfillment of the objectives of Part III, "Chemical Weed Control" on the HPR, Part II, research project "Research in Roadside Development". The Progress Report is also planned as a Technical Paper to be published. The authors are Drs. C. A. Lembi and D. J. Morre.

The report describes the isolation and partial characterization of a cell fraction that specifically binds a herbicide related to 2,4-D, called NPA. Virtually nothing is known about the initial events which lead to the selective killing action of 2,4-D and related herbicides. Previous studies have established that the herbicide must interact with a specific receptor molecule to initiate the killing action. But the nature of the elusive receptor molecule and its location within the cells of the plant were undecided. The results described in the enclosed report show that at least one herbicide receptor is localized on the surface membrane or plasma membrane of the plant cell. A method is described for isolating this particular membrane and for determining the amount of herbicide bound to it. In ongoing studies, these procedures are being used to determine the initial events in herbicide action and to learn more about the nature of the receptor molecule. This type of information is necessary to evaluate the safety and performance of existing herbicides as well as to develop new and more effective herbicides for the future.

The report is presented for acceptance as partial fulfillment of the objectives of the research and for approval of publication. As it is from an HPR project similar action by the ISHC and the FHWA will be requested.

Respectfully submitted,

Harold L. Michael

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Associate Director

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Purdue University
Lafayette, Indiana
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Summary. Plasma membrane-rich fractions were prepared from maize (Zea mays L.) coleoptiles by low shear homogenization and differential and sucrose gradient centrifugation. Plasma membrane fragments were identified using a specific cytochemical stain. In a comparison of 10 different cell fractions of varying plasma membrane content, the N-1-napthylphthalamic acid (NPA)-binding activity of the fractions was directly proportional to the content of plasma membrane. The NPA-binding appears to be strong (K_M between 10^{-8} - 10^{-7}M) but non-covalent. NPA is known to inhibit auxin transport efficiently and quickly. Thus, the results are consistent with the localization of auxin transport sites at the plant plasma membrane.

INTRODUCTION

Polar transport of auxin (indole-3-acetic acid or IAA) is a property long associated with grass coleoptiles (Went and Thimann, 1937). Indirect evidence suggests the location of auxin transport sites to be the plasma membrane (=plasmalemma) (e.g. Hertel and Flory, 1968; Leopold and dela Fuente, 1968; Osborne and Mullins, 1969; dela Fuente and Leopold, 1970). However, direct verification from in vitro analyses is lacking due to the absence of: 1) reliable procedures for fractionating plant cells and identifying isolated cell components and 2) specific and sensitive assay procedures for measuring membrane-associated binding or transport sites. This report concerns the application of recently developed procedures for isolation and identification of the plant plasma membrane (Lembi and Morré, 1970) in conjunction with a simple binding assay procedure to the problem of localizing specific binding sites within the plant plasma membrane. Studies on the specific binding of IAA itself did not yield clear results. Alternatively, binding of N-1-napthylphthalamic acid (NPA), a specific and rapidly acting inhibitor of IAA transport (Morgan and Soding, 1958; Hertel and Leopold, 1963; Hertel, Muller and Tavares, results unpublished) was used.

MATERIALS AND METHODS

Preparation of Cell Fractions. Maize (Zea mays L. var. WF-9 X M-14) coleoptiles, grown by method II of Morré, et al. (1967), were harvested 4 days after planting. Leaf rolls were removed. Approximately 30-40 g of tissue were homogenized in 120 ml of a freshly prepared medium consisting of 0.5 M sucrose, 0.1 M K_2HPO_4 and 0.02 M EDTA in coconut milk as the solvent and adjusted to pH 7.3 with NaOH. Nuclei and other endogenous cellular components of the coconut milk were removed by high speed centrifugation (100,000 X g, 90 min) before preparing the medium. Homogenates were prepared in the cold using a mechanized razor blade chopper (Morré, 1971). Cell walls and unbroken cells were removed by filtration through a single layer of miracloth (Chicopee Mills, New York), and a sample of the total homogenate was removed for determining NPA-binding activity. The remaining fraction was centrifuged for 30 min at 10,000 X g (Spinco L2-65B; SW27 rotor) to remove nuclei, plastids, mitochondria and large membrane fragments. The supernatant containing microsomes and the majority of the cytoplasmic membrane fragments was then top-loaded on a discontinuous gradient consisting of equivolume layers of 0.65, 0.8, 1.1, 1.2, 1.3 and 1.5 M sucrose in coconut milk prepared in a manner identical to the homogenization medium. Gradients were centrifuged for 90 min at 100,000 X g. Fractions were collected from the interfaces of the density layers, resuspended in homogenization medium and pelleted for 20-30 min at 100,000 X g.

For the experiments described in Table 2, a synthetic medium containing 0.08 M tris-acetate, pH 6.5, 0.025 M $MgCl_2$, 0.02 M $CaCl_2$, 0.05 M EDTA, 0.05 M KCl, 0.3 M sucrose and 4×10^{-5} M n-octanol was used. Coleoptiles were homogenized using a mortar and pestle in a ratio of 5 parts of tissue to 4 parts of medium.

Electron microscopy. Pellets obtained from aliquots of membrane suspensions or whole tissue explants were fixed in 2% buffered glutaraldehyde (0.1 M sodium phosphate, pH 7.3) for 18-20 hr at 4°. The material was rinsed with 3-5 changes of buffer over a period of 45 min and post-fixed in 1% buffered (0.1 M sodium phosphate, pH 7.3) osmium tetroxide for 1 hr at 26°. Specimens were rinsed thoroughly and dehydrated through a graded acetone series and embedded in epon (Luft, 1961).

Thin sections were stained with alkaline lead citrate (Reynolds, 1963) or subjected to a PTA-chromic acid procedure that is specific for the plant plasma membrane (Roland, 1969). To stain the plasma membrane, sections were floated over a 1% periodic acid solution for 30 min followed by 5 washes of 10 min each with distilled water. The sections were then treated with a mixture of 1% PTA and 10% chromic acid in distilled water for 5 min. After rinsing to remove excess stain, the sections were mounted on parlodion and carbon coated grids. Specimens were observed and photographed using a Philips EM 300.

Electron microscope morphometry. Quantitative estimation of plasma membranes were made on prints enlarged to 8 X 10 inches at magnifications of 25,000 according to the method of Loud (1962). A transparency having a grid of parallel lines spaced 1 cm apart was laid over the electron micrograph. An estimate of the amount of plasma membrane in isolated fractions was obtained from the number of intersections of the lines of the grid and the profiles or edges of dark-staining membranes of thin sections of pellets stained by the PTA-chromic acid procedure. Total membrane was determined by the same procedure from skipped sections of the same pellets after staining with lead citrate. Results are

expressed in terms of specific activity, i.e., the ratio of the number of plasma membrane intersections to the number of total membrane intersections.

NPA-binding assay. Unlabeled NPA (K-salt) was recrystallized from "Alanap-S" (U.S. Rubber Co., Naugatuck, Conn.). Labeled NPA was synthesized by Dr. J. E. Tavares, from equimolar amounts of 1-naphthylamine and ^{3}H-phthalic anhydride (general label; spec. act. 600 mC/mM, Radiochemical Centre, Amersham, England) mixed in boiling toluene. ^{3}H-NPA forms and crystallizes spontaneously. Material obtained in parallel with unlabeled phthalic anhydride was identified as NPA by infrared spectroscopy.

Supernatant and total homogenate fractions were tested directly. Pellet materials were loosened with a small pestle and resuspended in distilled water. Assays were conducted in two series at 4°. For series A, an aliquot (0.4 ml) of each fraction (0.3-3 mg protein) was mixed with ca. 0.025 ug ^{3}H-NPA (ca. 50,000 cpm) in distilled water with a final volume of 5 ml (^{3}H-NPA at 6 x 10^{-9}M). Series B was conducted in parallel in a manner identical to series A except that unlabeled NPA in distilled water was added to a final concentration of 10^{-5}M. Tubes from both series were centrifuged immediately and in parallel for 15 min at 30,000 X g. The supernatants were decanted, after which the tubes were drained carefully and wiped free of liquid. Pellets were carefully resuspended in 0.5 ml of water with disposable pipettes and transferred to scintillation vials. Bray's solution (10 ml) was added and radioactivity was determined using a Packard liquid scintillation spectrometer. Proteins were determined from separate aliquots by the method of Lowry et al. (1951). Radioactivity that was specifically bound to membrane fractions was determined as the difference between the sample in series A (total radioactivity) and the sample in series B (radioactivity not specifically bound).

RESULTS AND DISCUSSION

NPA is a very potent and rapidly acting inhibitor of auxin transport (Morgan and Soding, 1958; Hertel and Leopold, 1963; Hertel, Muller and Tavares, results unpublished), effective at concentrations as low as 10^{-7}M. At sufficiently high external concentrations, inhibition of IAA transport can be detected less than 1 min after NPA application. If auxin transport occurs at the plasma membrane (dela Fuente and Leopold, 1970; Hertel and Flory, 1968; Osborne and Mullins, 1969), the physiological effects of NPA on auxin transport and the in vitro binding of NPA to plasma membrane fractions should correlate in a significant way.

We first established that the plasma membranes could be specifically distinguished from other cytoplasmic membranes. In electron micrographs of coleoptile cells stained with lead citrate all cellular membranes stained intensely (Fig. 1). However, using the PTA-chromic acid procedure, only the plasma membrane was intensely stained (Fig. 2). Similar results were obtained with plant stems and roots (Roland, 1969; Morrè, et al.,1970).

The specificity of the PTA-chromic acid stain for plasma membrane is retained with isolated cell fractions, as reported previously (Morrè, et al., 1970). Problems of stain penetration into cells or fractions are eliminated since the procedure is applied to thin sections with the stain being equally accessible to all membrane surfaces. Figures 3 and 4 compare plasma membrane-containing fractions stained with lead citrate (Fig. 3) and by the PTA-chromic acid procedure (Fig. 4). Even with heterogeneous fractions, and in the absence of biochemical markers, amounts of plasma membrane can be accurately quantitated using techniques of electron microscope morphometry.

Based on the radioactivity recovered (93% of total), we estimate that approximately 40% of the membrane-associated NPA binding of the total homogenate was removed in the organelle pellet (10,000 X g) with the remaining 60% being retained in the 10,000 g supernatant fraction (Table 1). These results show that NPA binding was associated principally with some cell component other than nuclei, mitochondria or plastids. The NPA-binding fraction was concentrated in the 10,000 X g supernatant as were the plasma membrane fragments. Further fractionation of the cytoplasmic membranes of the 10,000 X g supernatant into various subfractions was achieved by sucrose density gradient centrifugation. Fractions of varying plasma membrane content (2-36%) and NPA-binding capacity (65-370 cpm/mg protein) were obtained (Table 1). As illustrated in Figure 5, a direct correlation between plasma membrane content and NPA-binding capacity was observed with a highly significant correlation coefficient ($r = 0.95$).

The highest proportion of plasma membrane in any fraction from maize coleoptiles was 36%. With stem tissues of onion and kohlrabi, fractions with greater concentrations ($<70\%$) of plasma membrane were obtained. However, these tissues do not yield cell fractions having the consistently high NPA-binding capacity associated with coleoptiles. The low yield of purified NPA-binding fraction also affected the results. Over 90% of the NPA-binding activity of the total homogenate was recovered in the 10,000 X g pellet and supernatant. Yet, on the average, only 12% (5-25%) of the total activity was recovered from the density gradients. This may be due to losses of coleoptile plasma membrane during sucrose gradient centrifugation.

NPA-binding activity of other cell components was ruled out on the following basis. Sucrose gradients yielded fractions enriched in mitochondrial, tonoplast, dictyosome and/or rough-surfaced endoplasmic reticulum fragments. These fractions did not bind NPA appreciably. Additionally, these cell components were absent or were present only as minor contaminants in fractions enriched in both plasma membrane and NPA-binding activity. On this basis, the only cell component consistently correlated with NPA binding was the plasma membrane. Even with the 10,000 X g pellet, an NPA-binding capacity of 51 $\pm$ 5 cpm/mg protein (Table 1) agreed with its plasma membrane content of 4% (cf. Fig. 5).

Unlabeled IAA at 10^{-5}, 10^{-4}, 10^{-3} and 3 X 10^{-3} M did not competitively inhibit ^{3}H-NPA binding. This suggests that the binding sites for auxin and NPA are different. Gibberellin A_3 and (+)-abscisic acid did not compete with ^{3}H-NPA binding at concentrations up to 10^{-3} M. Radioactivity was lost from material pelleted through a 1 cm deep layer of homogenate or pelleted in a medium in the absence of ^{3}H-NPA. These observations indicate that the binding of ^{3}H-NPA is non-covalent.

Results summarized in Table 2 demonstrate that specific binding sites are occupied at low concentrations of NPA. A high affinity of NPA for the binding sites is indicated (K_M approximately 2 X 10^{-8}M) as well as unspecific lower affinity binding (KI 10^{-7}M). Although, we do not resolve the relationship, if any, between the NPA site and the hypothetical IAA site, development of a technique for detecting the attachment of an auxin-like compound to a specific membrane structure is an important step toward understanding how plants respond to growth regulators of the auxin type.

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Table 1. Distribution of NPA-binding membranes among cell fractions from maize coleoptiles.

Fraction	Cpm/10 g fresh weight ± ave. dev.	% of total	Specific activity ± ave. dev. cpm/mg protein	Specific activity ± ave. dev. plasma membrane/ total membrane
Total homogenate	3027 ± 1162	100	_*	_*
10,000 X g pellet	1166 ± 750	38	51 ± 15	4 ± 1
10,000 X g supernatant	1673 ± 133	55	_*	_*
Plasma membrane fractions from sucrose gradient	362 ± 103	12	217 ± 110	21 ± 9

*Specific activities on a protein basis were not determined due to the contribution of proteins from the coconut milk contained in the isolation medium and present in the fractions.

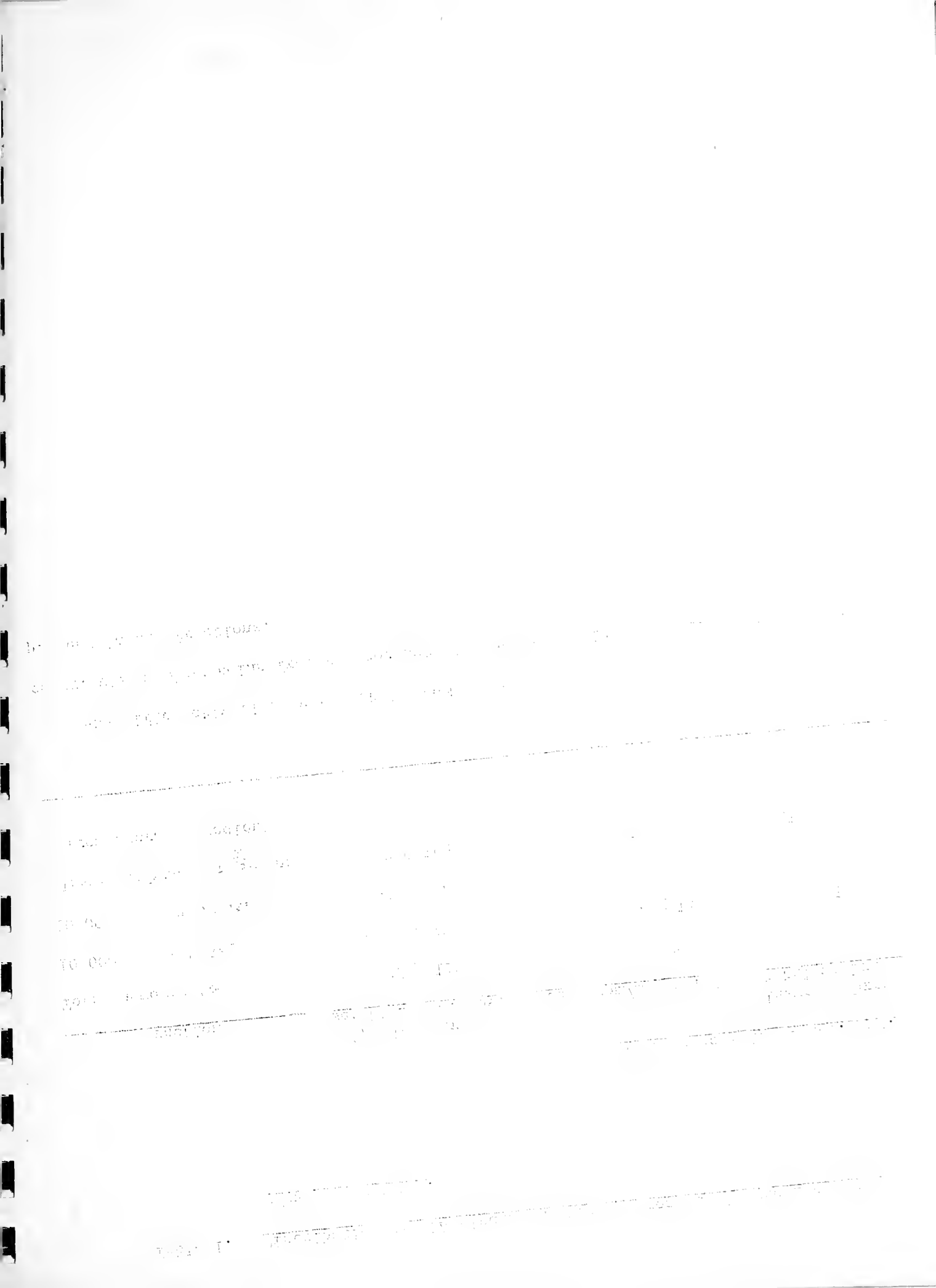


Table 2. NPA-binding as a function of NPA concentration*

Concentration of NPA	% NPA bound (= 100 x cpm in pellet / cpm in total sample)
5×10^{-10} M	27
10^{-9} M	30
3×10^{-9} M	24
10^{-8} M	20
3×10^{-8} M	13
10^{-7} M	11
3×10^{-7} M	7
10^{-6} M	4
10^{-5} M	4

*NPA (^{3}H-NPA plus unlabeled NPA) were added to 2 ml aliquots of the total homogenate which had been partially cleared by centrifugation for 10 min at 1500 X g. The aliquots were centrifuged for 20 min at 100,000 X g and the radioactivities of the pellets and of the supernatant were determined.

List of Figures

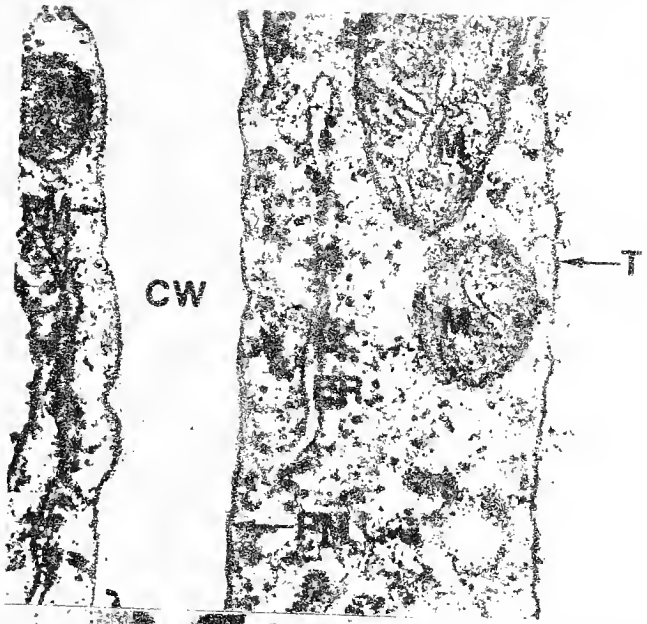
Fig. 1. Maize coleoptile tissue section stained with lead citrate. All membranes and organelles, plasma membrane (PM), tonoplast (T), mitochondria (M) and endoplasmic reticulum (ER), are intensely stained. The cell wall (CW) is lightly stained. X 49,000.

Fig. 2. Electron micrograph of maize coleoptile section treated with the PTA-chromic acid procedure. Except for occasional regions of the cell wall (CW), the plasma membrane is the only cellular component which stains darkly. Tonoplast (T), mitochondria (M), and endoplasmic reticulum (ER) are unstained. X 52,600.

Fig. 3. Electron micrograph of a cell fraction from the sucrose-coconut milk gradient. This preparation was section-stained with lead citrate and shows the numerous vesicles (V) and occasional mitochondrial fragments (M) which characterize the fraction. X 34,300.

Fig. 4. Plasma membrane is easily detected as darkly-staining vesicles (PM) in sections of isolated cell fractions which have been treated according to the PTA-chromic acid procedure. Other vesicles (V) and mitochondria (M) are unstained or only faintly visible. X 34,300.

Fig. 5. Regression line showing the relationship between plasma membrane content and the specific activity of NPA binding of cell fractions from the sucrose-coconut milk gradient. Different symbols indicate different experiments.



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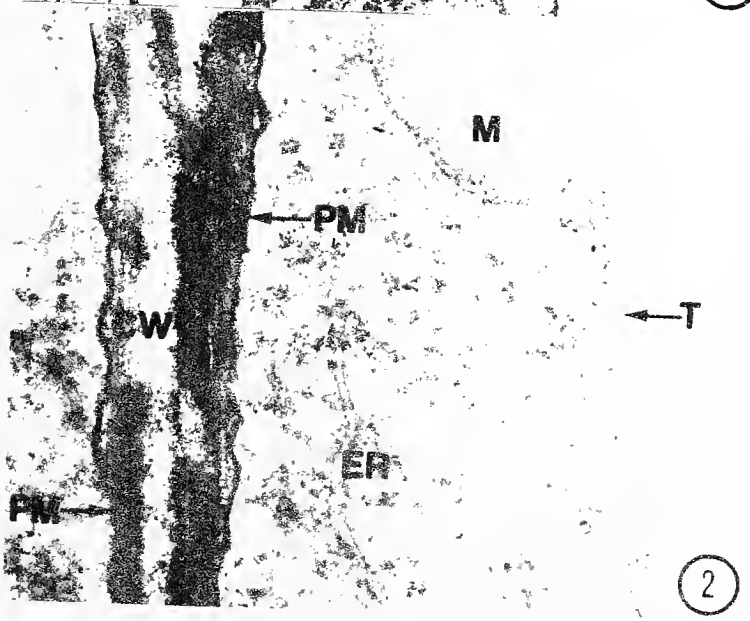

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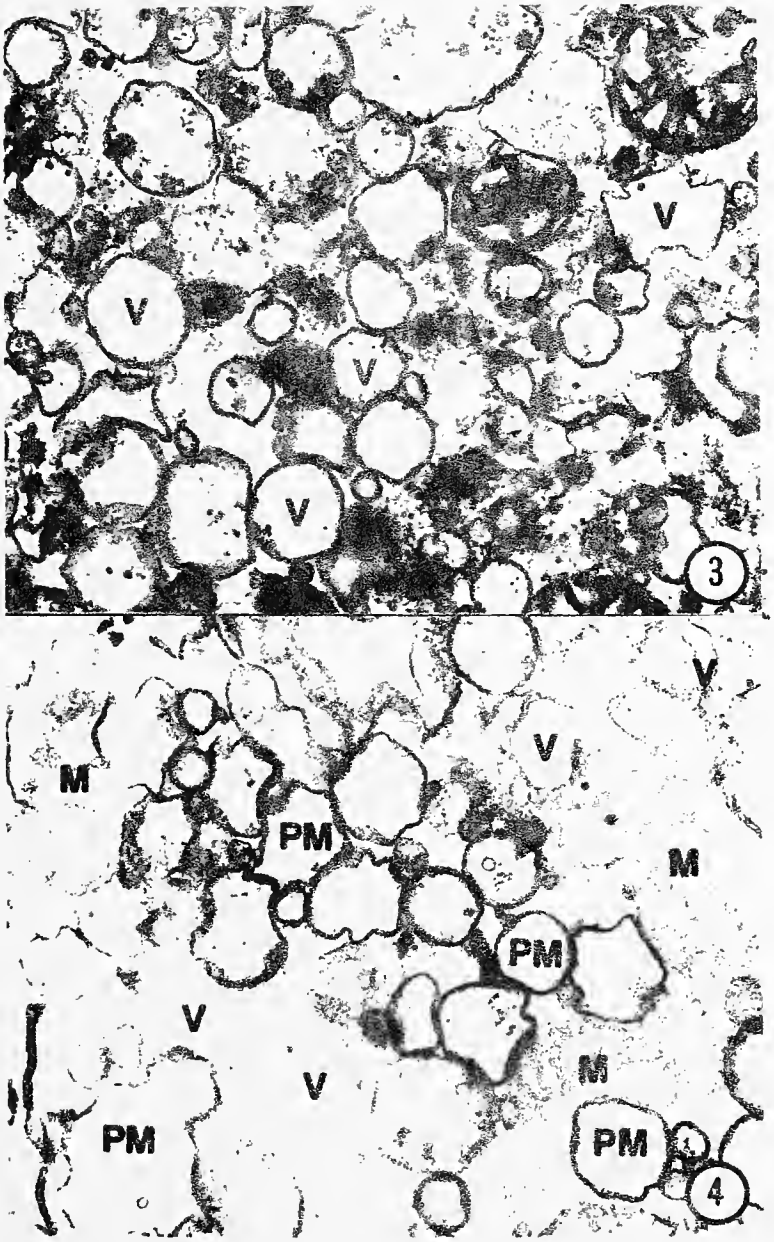
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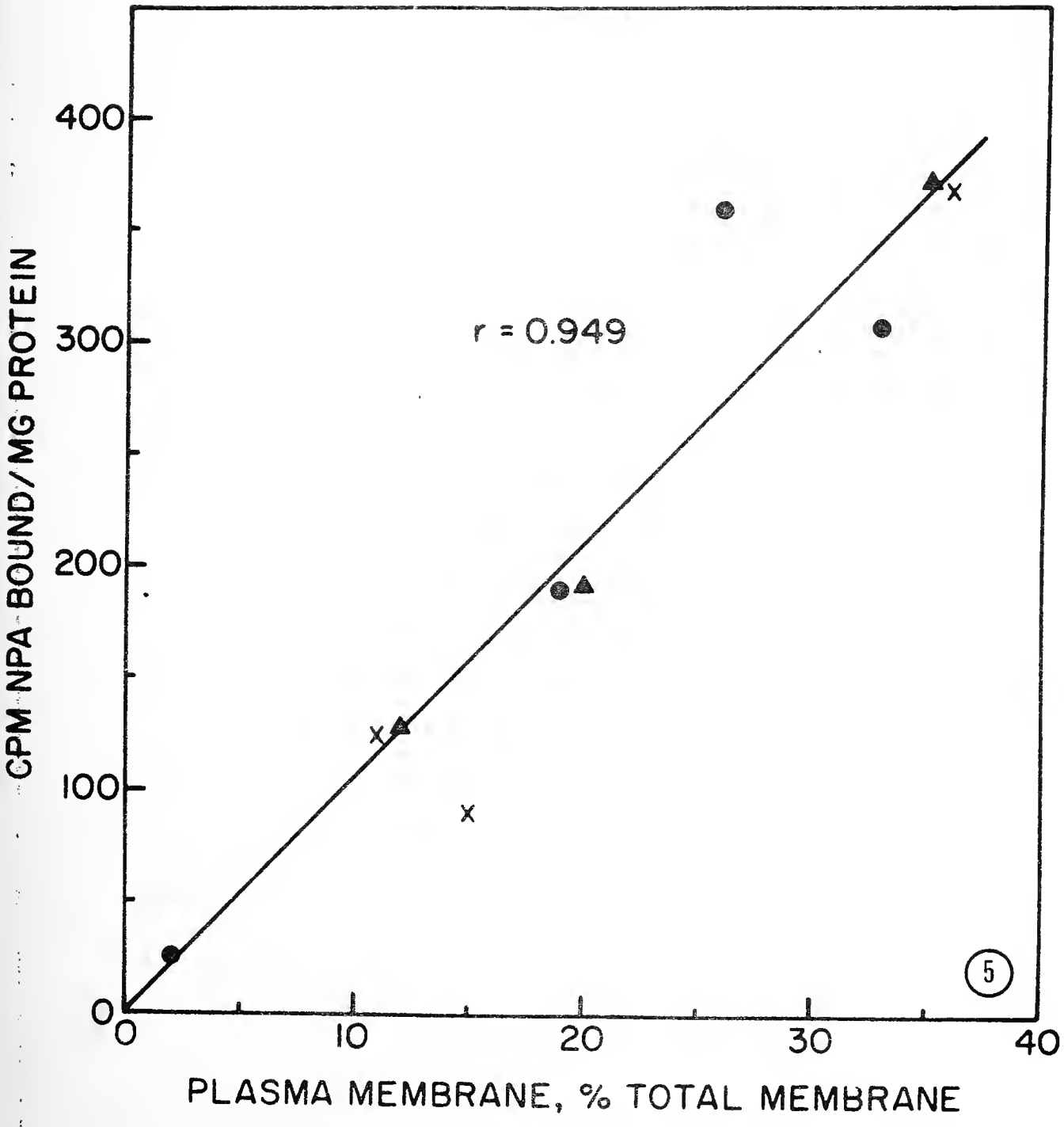
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r = 0.949
CPM NPA BOUND/MG PROTEIN
PLASMA MEMBRANE, % TOTAL MEMBRANE
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